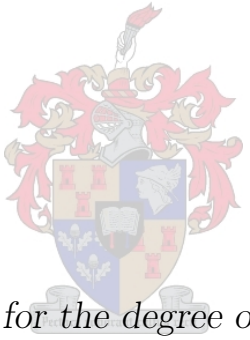
# Autophagic flux in neurodegeneration: Precision targeting with Spermidine and Rilmenidine

by

Dumisile Lumkwana



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# Abstract

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# Literature review

## 1.1 Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease and a leading cause of dementia in the elderly (Andrieu *et al.*, 2015). This disease is characterized by a progressive loss of synapses and neurons in certain brain regions (e.g. cerebral cortex and hippocampus), leading to impaired memory and deterioration of cognitive functions (DeKosky and Scheff, 1990; Scheff *et al.*, 2006; Zare-Shahabadi *et al.*, 2015), thus necessitating full-time medical care (Prince *et al.*, 2013). Currently the disease is incurable. Although a lot of efforts have been directed towards developing AD disease modifying therapies, current treatment strategies are aimed at ameliorating disease symptoms alone (Anand *et al.*, 2014; Di Santo *et al.*, 2013). Age is the most prominent risk factor for AD and about 44 million people are currently affected globally. While only 5% of individuals over the age of 65 years are affected by AD, the prevalence doubles with every 5 years of increasing age (Pimenova *et al.*, 2018; Qiu *et al.*, 2009). Given the rapidly aging population in first-and third- world countries, the prevalence of AD is predicted to rise to 81 million by 2025 (Alzheimer's Association, 2014; Ferri *et al.*, 2005). Moreover, it has been estimated that by 2050, 22% of the global population will be over the age of 60, with the majority residing in the developing nations (Annear *et al.*, 2015; Paddick *et al.*, 2013). In South Africa, there is limited statistics on the prevalence of AD. According to the census conducted in 2011, there are approximately 2.2 million people with some form of dementia. Very little is known about the prevalence of dementia and how it impacts on older adults residing in low and middle classes, as well as in the rural areas, where most of the adult population reside. Despite of this, it was reported that about 79% of patients were being cared for by family members (Kalula *et al.*, 2010). In the continued absence of effective therapeutic strategies to either delay or slow down the disease progression, AD will pose a tremendously burden on the healthcare and social services.

AD is a multifactorial disease that is highly complex, with genetic as well as and environmental causes (Dorszewska *et al.*, 2016).This disease is classified into two subsets.

The first being the early-onset Familial Alzheimer's disease (FAD, onset $< 65$), which contributes to the least cases of AD (1-5%) with strong genetic association (Musiek and Holtzman, 2015; Reitz and Mayeux, 2014; Swerdlow, 2007). The second being sporadic late-onset Alzheimer's disease (LOAD, onset $\geq 65$) which contributes to the majority of all AD cases ($> 95\%$), (Musiek and Holtzman, 2015; Reitz and Mayeux, 2014; Swerdlow, 2007), with an unclear cause (Dorszewska *et al.*, 2016; Pimenova *et al.*, 2018). Certain genes such as amyloid precursor protein (APP), Presenilin 1 (PSEN1) and Presenilin 2 (PSEN2) are responsible for occurrence of FAD, while APOE gene is responsible for LOAD (Dorszewska *et al.*, 2016).

AD pathology occurs in 5 stages; the pre-symptomatic, Mild Cognitive Impairment (MCI), mild AD, moderate AD and severe AD (Caldwell *et al.*, 2015). The first two stages encompasses a prodromal stage, which, in the majority of cases, precedes symptom onset by several years (at least 20 - 30 years) (Caldwell *et al.*, 2015; Caselli and Reiman, 2013; Penn *et al.*, 1993). During this time, pathological molecular changes occur inside the brain and are present as two molecular hallmarks which occur as a result of perturbations in cellular proteostasis. The senile plaques, which are extracellular deposits of amyloid beta ($A\beta$) peptides, and intraneuronal neurofibrillary tangles (NFTs), which are somatic inclusions of hyperphosphorylated, microtubule-associated protein tau, respectively (Mattson *et al.*, 2008) (Figure 1.1). To better understand the multifactorial pathophysiology of AD, several hypothesis have been put forward, including the amyloid cascade hypothesis (ACH), the cholinergic and tau hypothesis, as well as inflammation (Kurz and Perneczky, 2011); however, many molecular aspects and their dynamic changes during disease progression remain unclear. The ACH, the most widely accepted mechanistic hypothesis for AD, posits that an imbalance between the production and clearance of ($A\beta$) peptides is a very early, often initiating factor in disease onset (Hardy, 2009; Hardy and Higgins, 1992).

Proteolytic systems; the ubiquitin-proteasome system (UPS) and the lysyosomal systems [the autophagy-lysosomal pathway (ALP) and the endocytic- lysosomal pathway (ELP)] are responsible for the degradation of mis-folded or aggregated proteins in order to maintain cellular homeostasis. For example, the UPS targets and degrades short-lived proteins in the cytoplasm and nucleus, while the lysosomal system removes primarily long lived cytoplasmic proteins and damaged organelles (Ravikumar *et al.*, 2003; Rubinsztein *et al.*, 2005). A large body of evidence implicates these proteolytic systems in the AD pathophysiology. Dysfunction of these system has been well documented in AD pathophysiology and although extracellular $A\beta$ plaques and intraneuronal NFTs are defining hallmarks of AD neuropathology, a growing body of literature suggests that deficits in the autophagy–lysosomal pathway are likely to precede the formation of these pathological hallmarks (Cataldo *et al.*, 2000; Nixon and Yang, 2011; Perez *et al.*, 2015; Zare-Shahabadi *et al.*, 2015), suggesting a potential causality. More recently, a systems biology study highlighted the pivotal role of dysregulated autophagy in neurodegenerative diseases, where

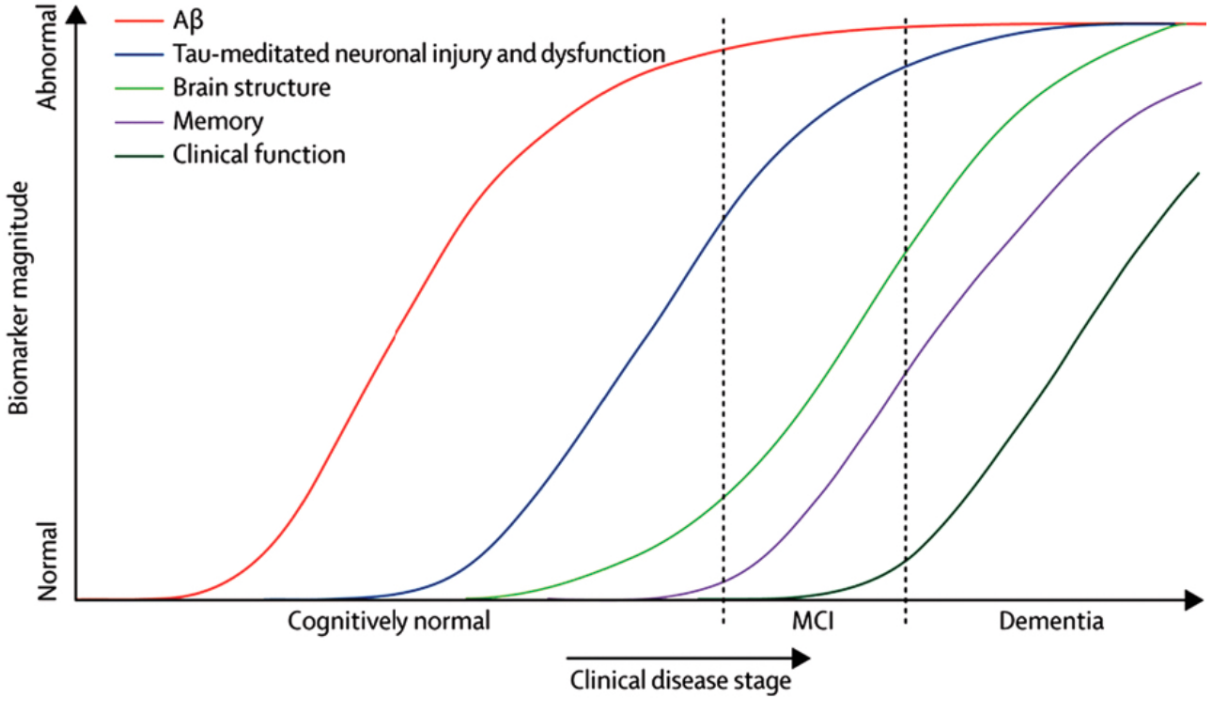


Figure 1.1: Hypothetical model of AD clinical disease stages in relation to biomarkers. $A\beta$ levels are deposited first and are the initial trigger AD while tau mediated neuronal injury manifests only late in the disease progression, ultimately leading neuronal death (Petrella, 2013) .

toxic protein aggregates and damaged organelles accumulate within specific types of neurons and lead to neuronal dysfunction and ultimately, demise (Caberlotto and Nguyen, 2014).

Although we have advanced our understanding of the molecular machinery that regulates the rate of protein degradation through autophagy at basal levels and the many aspects of its dysfunction in AD, the deviation of autophagic activity from basal levels and its change during disease pathogenesis in neuronal tissue remains largely unclear. Over the recent years, we have made substantial progress in modulating autophagy using pharmacological agents (Berger *et al.*, 2006; Hebron *et al.*, 2013; Ravikumar *et al.*, 2002, 2004; Rose *et al.*, 2010) or lifestyle interventions (Alirezaei *et al.*, 2010; Kuma *et al.*, 2004; Mizushima *et al.*, 2004; Scott *et al.*, 2004) in vitro and in vivo; yet many questions regarding an effective implementation of autophagy control remain unanswered. Therefore understanding the deviation of autophagic activity from basal levels and its change during disease pathogenesis as well as targeting or modulating autophagy activity precisely with the aim to restore autophagic flux may drive the development of better targeted therapeutic interventions that will slow down the disease progression and ultimately curing the disease instead of treating the symptoms. In this review, we start by describing neuronal metabolism and their metabolic profile and move to the involvement of reactive oxygen species in neurodegeneration, focusing on the role of oxidative stress and mitochondrial dysfunction in the pathogenesis of Alzheimer's disease. We then turn our focus to amyloid

beta biogenesis and its pathology in AD. We highlight the recent advances in the use super resolution techniques in molecular biology. We introduce autophagy and its molecular machinery and discuss its efficiency and essential function in neuronal cells, followed by its role in neurodegenerative diseases. We discuss how autophagic flux may differ in brain regions, and how the deviation of flux may relate to the state of protein aggregation in pathology. Furthermore, we review studies that localize the defect in autophagy in AD and assess the relationship between autophagic flux and neuronal cell death. Finally, we indicate the importance of accurately measuring and targeting autophagy and provide an overview of how autophagy may be modulated for therapeutic purposes in various model systems aimed at restoring autophagic flux.

## 1.2 Neuronal metabolism

A brain is made up of two cells types, neurons and astrocytes, which are highly interconnected and form functional networks through their spatial organization. The co-dependency of these cells types is reflected by their metabolic profiles where different yet complimentary pathways are used (Bélanger *et al.*, 2011; Schönfeld and Reiser, 2013) The brain has a high energy demand. This is evident by the fact that 20% of oxygen and 25% of the glucose utilized by the human body is dedicated to only cerebral function, yet the brain encompasses only 2% of the total body mass (Bélanger *et al.*, 2011). This means, uninterrupted supply of energy substrates from the circulation is required to meet the energy demands. Glucose is the main substrate of energy in the brain (Dienel, 2012; Pellerin and Magistretti, 2012) , however, other energy substrates such as lactate, pyruvate, glutamate, and glutamine can be utilized (Zielke *et al.*, 2009).

Glucose is delivered into a cell via specific glucose transporters (GLUTs) and once inside, it is phosphorylated by an enzyme called hexokinase (HK) to generate glucose-6-phosphate (glucose-6P) (Bélanger *et al.*, 2011; Herrero-Mendez *et al.*, 2009). The latter can enter three main metabolic pathways; glycolysis, pentose phosphate pathway (PPP), and glycogenesis in order to produce adenosine triphosphate (ATP). In glycolysis, glucose-6P is metabolized to produce pyruvate, 2 ATP molecules and NADH (Bélanger *et al.*, 2011). Pyruvate can be metabolised further in the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OxPhos) in the mitochondria using oxygen to generate 30-34 ATP molecules and CO2 or it can be reduced to lactate by lactate dehydrogenase (LDH) in the cytosol (Bélanger *et al.*, 2011). In PPP, glucose-6P is metabolized to generate NADPH, while it is stored as glycogen in glycogenesis, with the latter occurring in astrocytes (Bélanger *et al.*, 2011) (Figure 1.2). More importantly, astrocytes and neurons have the ability to oxidized glucose and/or lactate (Zielke *et al.*, 2009).

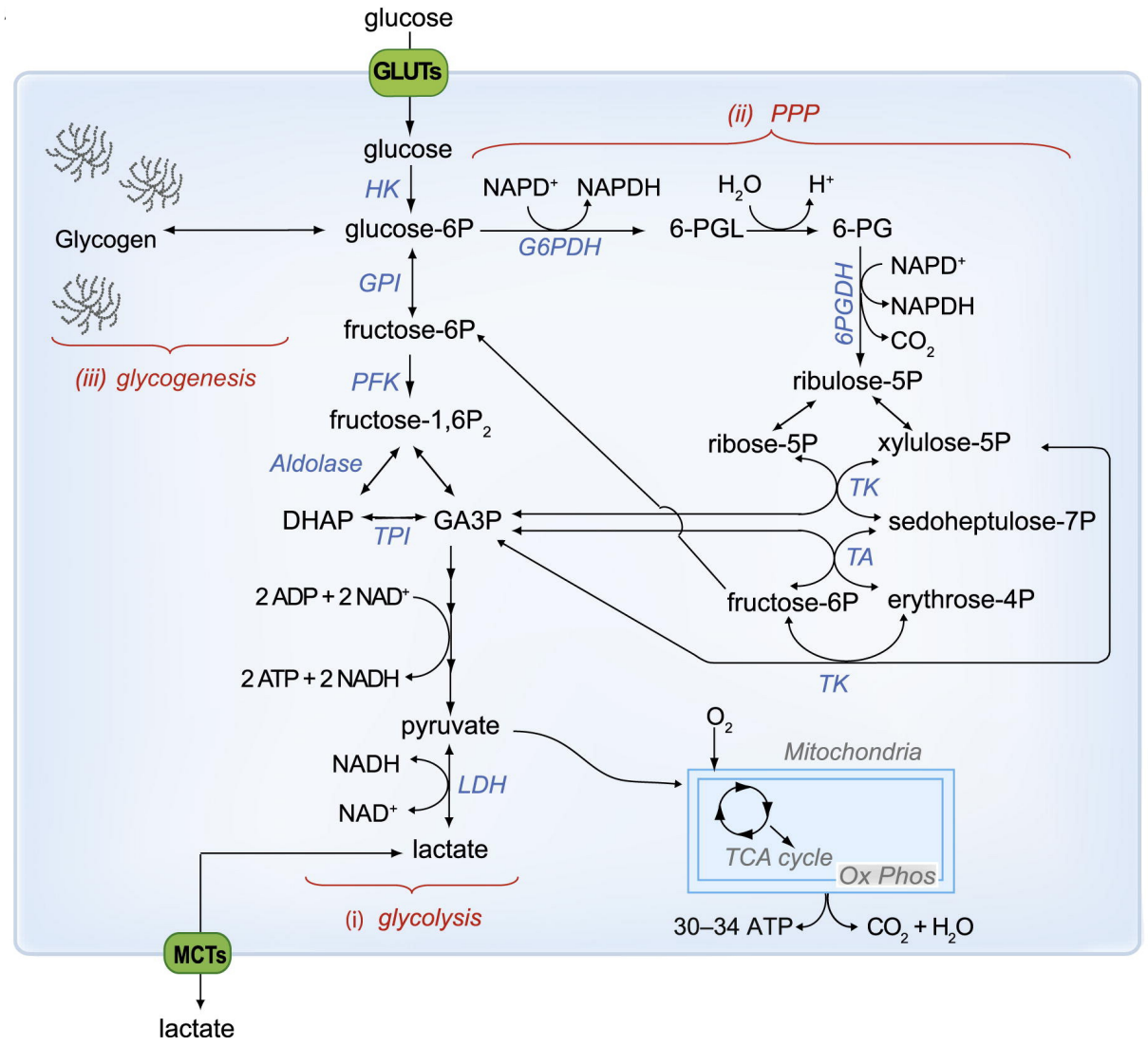


Figure 1.2: Glucose metabolism. A schematic diagram showing three main pathways; glycolysis, pentose phosphate pathway, and glycogenesis where glucose can be metabolized (Bélanger *et al.*, 2011) .

## Metabolic profile of neurons

Neurons are post-mitotic, highly differentiated cells that are characterized by high energy demands. This is because of their high levels of protein synthesis, which consumes high amount of ATP within the mammalian cells (Buttgereit and Brand, 1995). Neurons depend almost exclusively on the energy produced through OxPhos (30 - 34 ATP molecules) compared to glycolysis (2 ATP) in order to meet their high energy demand needed to perform cellular functions, such as synaptic plasticity and neurotransmitter synthesis (Cenini *et al.*, 2019; Mattson *et al.*, 2008; Schönfeld and Reiser, 2013). Mounting evidence demonstrate that neurons are capable of using lactate as an energy substrate (Boumezbeur *et al.*, 2010; Bouzier *et al.*, 2000; Serres *et al.*, 2005) and prefer lactate over glucose when both substrates are available (Bouzier-Sore *et al.*, 2006; Itoh *et al.*, 2003). Thus the specific characteristics of neurons are probably underly their distinct

metabolic profile. For example, glycolytic enzyme 6-phosphofructose-2-kinase/fructose-2, 6-bisphosphatase-3 (PFKFB3) is highly expressed in astrocytes, but virtually absent in neurons because of a constant proteasomal degradation (Almeida *et al.*, 2004; Herrero-Mendez *et al.*, 2009). Because of this, neurons unlike astrocytes display a lower glycolytic rate, and thus cannot upregulate this pathway in response to cellular stress (Almeida *et al.*, 2004; Herrero-Mendez *et al.*, 2009). Indeed, a previous study showed that upregulation of glycolysis via PFKFB3 in neurons is in fact detrimental, resulting to oxidative stress and apoptosis (Herrero-Mendez *et al.*, 2009). In this study, it was though that the upregulation of glycolysis occurs at a cost of PPP metabolism that is needed to produce NADPH, an antioxidant vital for maintaining cellular redox state (Herrero-Mendez *et al.*, 2009). Evidently, it has been shown that neurons have low NADPH compared to astrocytes (Ben-Yoseph *et al.*, 1996; García-Nogales *et al.*, 2003), and since antioxidant system and the related enzymes are important for maintaining neuronal integrity and survival by keeping the levels of reactive oxygen species (ROS) relatively low (Cenini *et al.*, 2019), it is not surprising that neurons are vulnerable to oxidative damage, implicated in neurodegeneration

## 1.3 ROS and its role in neurodegeneration

ROS are a group of reactive molecules that produced naturally in biological systems as part of normal cellular metabolism and are important in maintaining cellular homeostasis (Cenini *et al.*, 2019). These include superoxide ($O_2^-$), hydroxyl radical (·OH), hydroxyl ion ($OH^-$) and hydrogen peroxide ($H_2O_2$), all of which are generated from oxygen. $O_2^-$ is generated from O2 in the mitochondria as a result of the respiratory chain complex or NADPH oxidase and can be converted by superoxide dismutase (SOD) enzyme to produce $H_20_2$. The later in turn can be converted to other types of ROS, for example OH and $OH^-$ (Kim *et al.*, 2015), with ·OH being the most reactive ROS responsible for cytotoxicity (Bolisetty and Jaimes, 2013).

Under physiological conditions, ROS are maintained at relatively low levels by antioxidant system (Dasuri *et al.*, 2013; Gandhi and Abramov, 2012), and are involved cellular processes such as inflammation, immune response, cell survival, synaptic plasticity, learning, and memory (Cenini *et al.*, 2019; Kishida and Klann, 2007; Liu *et al.*, 2017). However, increased ROS production can be harmful because of its ability to oxidise nucleic acids, protein and lipids (Wang *et al.*, 2014). Increased ROS accumulation has been implicated in oxidative stress, mitochondrial dysfunction and in gliosis. However, role of ROS in gliosis is poorly understood and only one study provides evidence (Kishida and Klann, 2007).

Excessive accumulation of ROS due failure of antioxidant system or increased ROS production can result in oxidative stress, an imbalance between rate of ROS production

and clearance (Wang *et al.*, 2014). High levels of oxidative stress have been implicated in aging and in the pathogenesis of various neurodegenerative diseases (Bonda *et al.*, 2010; Cenini *et al.*, 2019; Liu *et al.*, 2017; Shibata and Kobayashi, 2008). Neurons are mostly susceptible to oxidative stress and damage due to its high oxygen consumption, high energy demand, low antioxidant defenses as well as high abundance of polyunsaturated fatty acid which are susceptible to lipid peroxidation (Cobley *et al.*, 2018). Thus, it is not surprising that ROS induced oxidative damage is widely reported in AD. In addition, since mitochondria are the major source of ROS production and the main target of oxidative stress, progressive mitochondrial dysfunction has also been implicated in the pathogenesis of AD (Swerdlow, 2007). The involvement of oxidative stress and mitochondrial damage in AD has been demonstrated in different models and are described below.

## Evidence of oxidative stress in AD

Oxidative damage is one of the earliest events in AD (Nunomura *et al.*, 2001). This is supported by several studies that demonstrated elevated levels of oxidative stress in patients with mild cognitive impairment (MCI) (Ansari and Scheff, 2010; Praticò and Sung, 2004; Williams *et al.*, 2006). In addition, antioxidants including uric acid, vitamin C and E as well as antioxidant enzymes such as superoxide dismutase (SOD) were found to be decreased in MCI patients (Rinaldi *et al.*, 2003; Torres *et al.*, 2011). Increased oxidative stress has also been implicated in AD. Excessive production of ROS is thought to play an essential role in the accumulation and deposition of $A\beta$ peptides (Bonda *et al.*, 2010). Ferreiro *et al.* (2008) reported that $A\beta$ plaques depleted $Ca^{2+}$ ions storage in the endoplasmic reticulum (ER), leading to in cytosolic $Ca^{2+}$ overload, which resulted in the reduction of endogenous GSH levels and ROS accumulation of ROS. In addition, increased $H_2O_2$ levels and increased peroxidation of proteins and lipids were shown in transgenic mouse expressing APP/PS-1, suggesting that $A\beta$ may exacerbate oxidative stress in AD (Matsuoka *et al.*, 2001; Zhao and Zhao, 2013). In addition, products of lipid peroxidation such as 4-hydroxynonal (4HNE), malondialdehyde (MDA), and 2 propenal (acrolein) have been found to be elevated in multiple studies performed in patient with AD (Wang *et al.*, 2014; Zhao and Zhao, 2013). For example, significantly increased levels of 4HNE were reported in the hippocampus (Lovell *et al.*, 1995; Markesbery and Lovell, 1998; Montine *et al.*, 1998), parahippocampal gyrus (Markesbery and Lovell, 1998), entorhinal and temporal cortex (Montine *et al.*, 1998), amygdala (Lovell *et al.*, 1995; Markesbery and Lovell, 1998), ventricular fluid (Lovell *et al.*, 1997), and plasma (McGrath, 2001) in AD patients versus control subjects of the same age. Similar findings were observed with MDA and acrolein in AD patients. MDA was found to be increased in the hippocampus (Lovell *et al.*, 1995), pyriform cortex (Lovell *et al.*, 1995), temporal cortex (Marcus *et al.*, 1998; Palmer and Burns, 1994) and occipital cortices (Miranda *et al.*, 2000), while elevated levels of acrolein were reported in the hippocampus/parahippocampal gyrus (Bradley

*et al.*, 2010; Calingasan *et al.*, 1999; Lovell *et al.*, 2001; Williams *et al.*, 2006), amygdala (Lovell *et al.*, 2001),superior and middle temporal gyri (Bradley *et al.*, 2010; Williams *et al.*, 2006), and cerebellum (Bradley *et al.*, 2010; Williams *et al.*, 2006). Altogether, these studies demonstrate the involvement of oxidative stress in AD

## Evidence of mitochondrial dysfunction in AD

As previously mentioned, mitochondria are the main source of oxidative damage due to the continuous generation of superoxide anion that results from electron leakage during electron transfer. Although mitochondria have an efficient antioxidant system, the production of superoxide anions is responsible for 90% of the endogenous ROS (Wang *et al.*, 2014). It has been suggested that impaired mitochondria are more efficient producers of ROS, but less efficient producers of ATP. In fact, reduced energy metabolism in the brain is among one of the well documented abnormalities in AD (Wang *et al.*, 2014). A genome-wide transcriptomic study suggested that decreased cerebral glucose metabolism in AD was associated with reduced expression of neuronal genes encoding subunits of the mitochondrial electron transport chain. In support of this, several studies reported reduced expression of $\alpha$-ketoglutarate dehydrogenase complex, pyruvate dehydrogenase complex, and cytochrome oxidase in AD, which are the key enzymes of oxidative phosphorylation (Chandrasekaran *et al.*, 1994; Cottrell *et al.*, 2001; Maurer *et al.*, 2000). Due to the role of mitochondria in calcium homeostasis, previous studies reported calcium mishandling i.e. increased calcium overload and decreased reuptake of calcium in fibroblasts of patients with AD (Ito *et al.*, 1994; Peterson *et al.*, 1985). Area-Gomez *et al.* (2012) reported a significant increase in function of mitochondria-associated ER membranes and ER–mitochondrial communication, measured by cholesteryl ester and phospholipid synthesis, respectively in patients with familial and sporadic forms of AD (Area-Gomez *et al.*, 2012). Consistent with this, ER–mitochondria interface proteins were found to be highly expressed in early stage of AD in patients with AD as well as in the mouse model of $APP_{Swe/Lon}$. Lastly, elevated oxidative damage to mitochondrial DNA was reported in patients with AD (Mecocci *et al.*, 1994; Wang *et al.*, 2005). Taken together, these studies provide evidence of the involvement of mitochondrial dysfunction in AD.

## Role of PQ in oxidative stress and in the pathogenesis of AD

PQ is a pesticide that is widely used globally for agricultural practices. Several epidemiological studies have reported that pesticide exposure increases the risk of developing AD (Baldi *et al.*, 2003; Hayden *et al.*, 2010; Santibañez *et al.*, 2007; Yan *et al.*, 2016). In fact, genetic factors and environmental factors are the aetiology of the sporadic form of AD (Landrigan *et al.*, 2005). Although the mechanism of PQ are better understood PD than in AD, it is known that PQ accumulates in the cerebral cortex and hippocampus (Landri-

gan *et al.*, 2005). Therefore, PQ could potentially impair learning and memory functions and affect AD pathogenesis. PQ exact its toxicity by inducing oxidative stress and mitochondrial damage (Baltazar *et al.*, 2014; Drechsel and Patel, 2008; Lin and Beal, 2006), both of which are implicated in the pathogenesis of AD (Lin and Beal, 2006). Therefore, experimental models with PQ are widely used for understanding the roles of pesticide exposure in the development of AD as well as to study the mechanisms of AD. Drechsel and Patel (2008) showed that PQ enhances $H_2O_2$ production in brain mitochondrial. Since $H_2O_2$ is essential for regulating redox-sensive signaling under normal condition (Rhee, 2006), it is not surprising that its increased generation from the mitochondria has been implicated in the development of AD (Du *et al.*, 2008; Manczak *et al.*, 2006). In another study, exposure to PQ was found to increase oxidative stress measured with 4HNE and nitrotyrosine levels in the mitochondria of cerebral cortex and to exhibit mitochondrial dysfunction in wild-type mice and APP transgenic mice (Chen *et al.*, 2012). The authors also reported an increase in mitochondrial damage which was found to be directly correlated with impaired learning and memory as well as elevated $A\beta$ levels (Chen *et al.*, 2012). Moreover, it was reported that overexpression of peroxiredoxin 3, a mitochondrial antioxidant defense enzyme, whose role is to remove $H_2O_2$ in the mitochondria protected against PQ induced mitochondrial damage, while decreasing $A\beta$ levels and improving cognition (Chen *et al.*, 2012). These results suggest the importance of $H_2O_2$ in the pathogenesis of AD.

## 1.4 $A\beta$ biogenesis

Amyloid precursor protein (APP), an evolutionary conserved type 1 transmembrane protein, is unequivocally linked to AD pathogenesis as the unique source of neurotoxic forms of $A\beta$ (Chen *et al.*, 2015; Rajendran and Annaert, 2012). During early development, APP is highly enriched at the growth cones of developing neurites (Ramaker *et al.*, 2016; Sabo *et al.*, 2003). In more mature neurons, APP localizes to focal adhesion sites and within pre- and postsynaptic structures of the central and peripheral nervous tissue, suggesting a functional role in neuritic growth and synaptic plasticity (Ashley *et al.*, 2005; Yamazaki *et al.*, 1997). APP is synthesized in the ER and transported to the Golgi apparatus where it is packaged into vesicles for delivery to the cell surface for further processing by $\alpha$-, $\beta$-, and $\gamma$-secretases following the non-amyloidogenic (constitutive) or amyloidogenic pathway (Figure 1.3) (O'Brien and Wong, 2011; Ramaker *et al.*, 2016). The cleavage activity of the $\beta$, and $\gamma$-secretases is mediated by the $\beta$-site APP-cleaving enzyme 1 (BACE1) and presenilins (PSENs) catalytic domain, respectively (Rajendran and Annaert, 2012).

The non-amyloidogenic pathway leads to the production of non-pathogenic fragments, while the amyloidogenic pathway promotes the generation of $A\beta$ peptides. Briefly, following the former pathway, APP is first cleaved by $\alpha$-secretase also known as distintegrin

or metalloproteinase 10 (ADAM10), within the $A\beta$ sequence, thereby blocking $A\beta$ production, to generate two proteolytic fragments: soluble APP$\alpha$, and the corresponding C-terminal fragments, $\alpha$-CTF/C83 (a protein stub that remains secured to the plasma membrane for further proteolytic processing) (Gandy *et al.*, 1994; Roychaudhuri *et al.*, 2009). Soluble APP$\alpha$ is recycled back to the cell surface by the recycling compartments or delivered to the lysosome for degradation through the endosomal– lysosomal system (Caster and Kahn, 2013; Golde *et al.*, 1992). In the amyloidogenic pathway, APP is cleaved by $\beta$-secretase, the major secretase in the brain, at the N-terminus of the $A\beta$ sequence, thus generating soluble APP$\beta$, which is released extracellularly, and the corresponding C-terminal fragment, $\beta$-CTF/C99 (a membrane-associated fragment comprising the entire $A\beta$ sequence). Both C99 and C83 are subsequently cleaved by $\gamma$-secretase within the transmembrane domain, resulting in the release of a nontoxic p3 fragment, APP intracellular domain, and $A\beta$ peptide species of slightly different lengths (Figure 1.3) (Cole and Vassar, 2007; Jarrett *et al.*, 1993). Secretase cleavage gives rise to an admixture of $A\beta$ peptides composed of 39–43 amino acids, with $A\beta40$ (90%) and $A\beta42$ (10%) being the two major $A\beta$ species (Gouras *et al.*, 2000; Takahashi *et al.*, 2013). Neuronal cells produce both $A\beta40$ and $A\beta42$ peptides, with healthy neurons having a high $A\beta40/A\beta42$.

## Role of $A\beta$ pathology in AD

Although $A\beta42$ is produced at low quantities in neurons, it has a higher tendency to self-aggregate and form higher order structures, including toxic $A\beta$ dimers, trimers, and oligomers. These higher order structures able to coalesce to form fibrils in insoluble beta-sheet conformation that eventually deposit into diffuse senile plaques (Burdick *et al.*, 1992; Gravina *et al.*, 1995). However, various studies have shown that the oligomers are main source of its neurotoxicity (Shankar *et al.*, 2008; Shankar and Walsh, 2009). Although autophagy is responsible for the bulk degradation of aberrant proteins, not all aggregate-prone proteins are fully amenable to autophagic degradation (Wong *et al.*, 2008). For example, expression of human $A\beta40$ and $A\beta42$ in Drosophila brain has been shown to have differential effects on neuronal autophagic degradation Ling2009. Studies have shown that although autophagy sequesters $A\beta42$, this aggregate-prone peptide in turn may decrease the degradative capacity of autophagy (Ling *et al.*, 2014; Ling and Salvaterra, 2011). This was demonstrated by highly concentrated intracellular $A\beta$ identified in autophagic vacuoles (AVs), which accumulate in affected neurons, especially with advancing age (Ling and Salvaterra, 2011). In contrast, sequestration of $A\beta40$ does not produce any detectible changes in either the neuronal autophagy activity or neurological defects in vivo, which is consistent with the ACH for AD pathogenesis (Hardy and Higgins, 1992).

Prior to senile plaque deposition, $A\beta42$ oligomers are able to induce oxidative damage, promote tau hyperphosphorylation, and lead to synaptic and mitochondria toxicity (Figure 1.3) (Kaminsky *et al.*, 2015; Lustbader *et al.*, 2004). Moreover, during late dis-

ease progression, $A\beta 42$ senile plaques have been found to activate microglia (Rosenmann, 2013). Microglial activation results in the production and release of proinflammatory cytokines, including IL-1$\beta$, TNF-$\alpha$, and IFN $\gamma$, which in turn stimulate the nearby astrocytes to further exacerbate $A\beta 42$ production and dispersal (Prà *et al.*, 2015). To this end, immunohistochemical analysis has revealed significantly higher $A\beta 42$ levels in AD brains than control brains (Funato *et al.*, 1998). Additionally, the extent of $A\beta 42$ deposition is much greater in AD brains with disease progression, while $A\beta 40$ shows little or no apparent age-dependent accumulation (Funato *et al.*, 1998).

It is well established that AD-causing mutations in APP and in presenilin 1 (PSEN1) and presenilin 2 (PSEN2) alter APP proteolytic processing in a manner that alleviates the relative levels of the $A\beta 42$ peptides (Borchelt *et al.*, 1996; Scheuner *et al.*, 1996). Mutations in APP that lie within the $A\beta$ sequence increase the self-aggregation of the resultant peptides, not their production, while mutations in PSEN1 and PSEN2 increase the relative production of the longer, more hydrophobic, and self-aggregating $A\beta 42$ peptides (Kim and Kim, 2008; Weggen and Beher, 2012). Furthermore, the inactivation of PSEN1 and PSEN2 has been shown to completely prevent $A\beta$ generation (Herreman *et al.*, 2000; Zhang *et al.*, 2000). Although $A\beta 42$ is generated at a 10-times lower rate than $A\beta 40$, the former peptide has consistently been shown to be the main component of $A\beta$ plaques in AD (Iwatsubo *et al.*, 1994). Originally, the ACH was mostly driven by genetic studies indicating the vast majority of early-onset familial AD mutations to confer a similar biochemical phenotype, i.e., an increased ratio of cerebral $A\beta 42$, either through an increased $A\beta 42$ production or decreased $A\beta 40$ production, or a combination of both (Cavallucci *et al.*, 2012; Cruts and Van Broeckhoven, 1998). And although the ACH takes a central position in AD-related research, the prevailing hypothesis does not entirely account for the complex pathophysiology of AD. Instead it seems that the role of $A\beta$ in synaptic degeneration may act in concert with several other factors that impair the integrity of neuronal functions (Anand *et al.*, 2014; Prà *et al.*, 2015). Growing evidence supports that dysregulated production of both $A\beta$ and tau may synergistically disrupt synaptic activity and mitochondrial function, resulting in AD (Chételat, 2013; Musiek and Holtzman, 2015; Quintanilla *et al.*, 2012; Teplow, 2013). Although many factors contribute to AD pathogenesis, imbalance in $A\beta$ production and clearance has emerged as the most extensively validated and compelling therapeutic target for both genetic and sporadic AD, as both forms of the disease can be ascribed similar etiologies (Selkoe, 2012; Selkoe and Hardy, 2016).

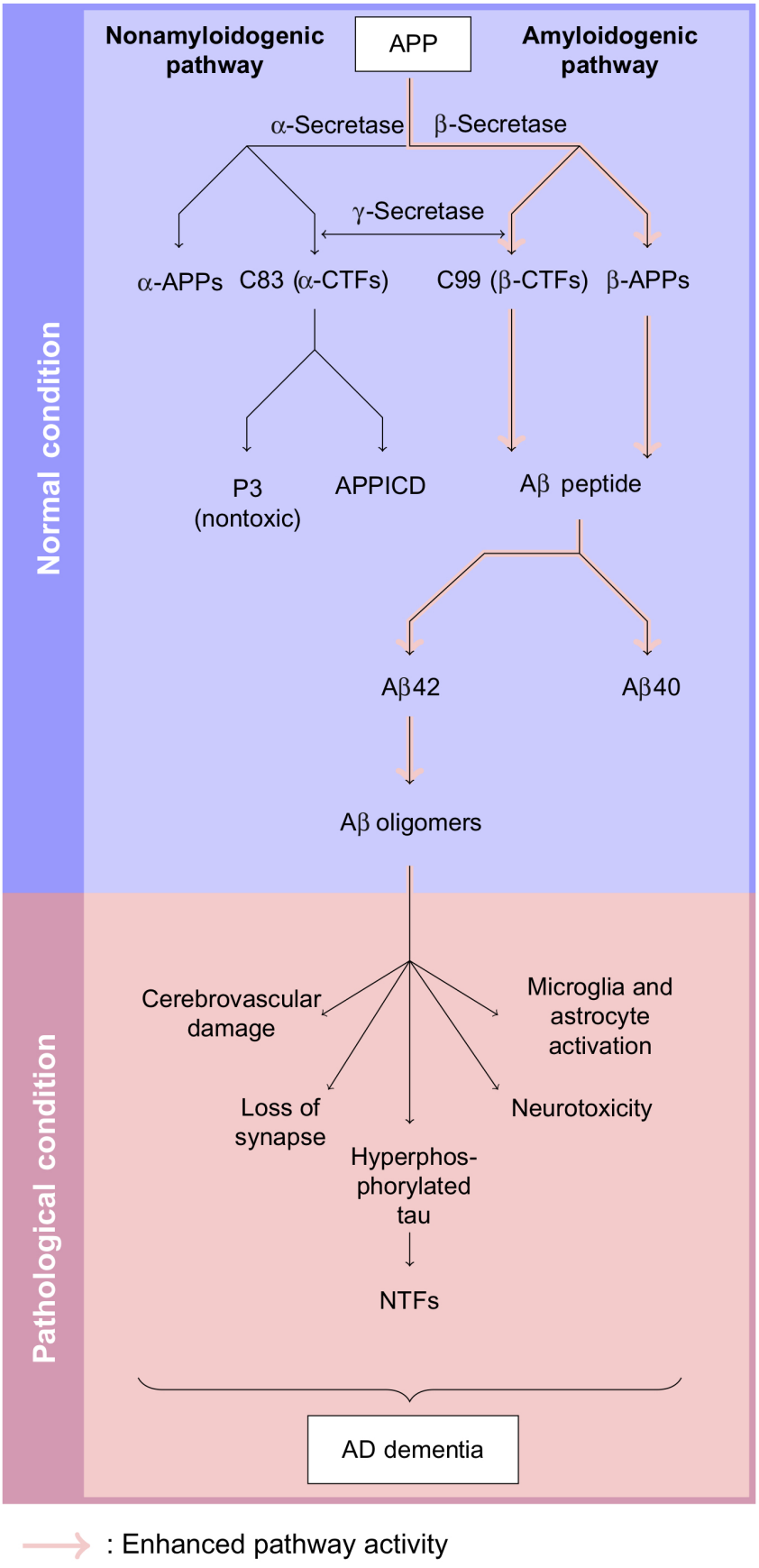


Figure 1.3: The nonamyloidogenic and amyloidogenic pathways. Enhanced amyloidogenic pathway activity increases neuronal synthesis of aggregate prone toxic $A\beta$ oligomers, which in turn lead to autophagy and mitochondrial dysfunction as well as tubulin disruption, thereby driving neurofibrillary tangle (NFT) formation

# Chapter 2

# Methodology

## 2.1 Introduction

# Chapter 3

# Autophagic flux assessment

## 3.1 Introduction

Autophagy is a physiological cellular process that is characterized by a fine balance between the rate of AV formation and the rate of their clearance, with autophagic flux referring to the rate of flow of material through the autophagic pathway (Loos *et al.*, 2014; Klionsky *et al.*, 2016). Many tools and techniques are available to assess autophagy, including western blotting (WB), fluorescence microscopy (FM), Transmission Electron Microscopy (TEM), flow cytometry (Klionsky *et al.*, 2016) and Correlative Light and Electron Microscopy (CLEM) with each technique having particular advantages, but also inherent disadvantages.

Autophagy plays a significant role in health where primarily decreased activation of autophagy has been shown to have detrimental effects. Autophagy can be induced via mTOR dependent and mTOR independent pathways (Sarkar, 2013). Although we have progressed substantially in the understanding of the autophagy machinery and its regulation, its dysfunction in pathology as well as its dynamic changes however in the disease progression remains often largely unclear. Furthermore, although a substantial progress has been made in unravelling the role and regulation of autophagy and its modulation in neurodegenerative disease using pharmacological agents, major differences exist between experimental design, drug concentrations used, duration of treatment intervention as well as the model system used to assess autophagic flux (Lumkwana *et al.*, 2017). This contributes to inability to modulate autophagy with high precision. Specifically, spermidine and rilmenidine have been shown to induce autophagy, but the impact of concentration ranges on autophagic flux and on subsequent protein clearance and neuronal toxicity is unclear. Importantly, whether a concentration dependent effect on autophagy activity exists achieving defined, but distinguishable heightened autophagy flux remains unclear. Particularly in terms of fine-tuning autophagy activity with a given flux deviation in neurodegeneration, this aspect is of critical importance. In this chapter we firstly aimed to establish a suitable concentration of spermidine (Spd) and rilmenidine (Ril) using GT

1-7 neuronal cells that induces autophagic flux without causing cellular toxicity, and secondly, to characterize the autophagic flux profile of a low and high concentration of both drugs established in terms of (1) the autophagic machinery and pathway intermediates (autophagosomes, autolysosome),(2) the autophagosome flux, (3) p62 puncta, and (4) autophagic vacuoles (AVs). For this purpose, GT1-7 neuronal cells were treated with three concentrations of Spd and Ril in the presence and absence of BafA1. Subsequently, cellular viability and WB for LC3 II and p62 was performed. Next, to assess the effect of a low and high concentration of Spd and Ril, GT 1-7 cells were treated with 1 & 10.0 $\mu$M respectively in the presence and absence of BafA1 and thereafter extensively analysed using FM and TEM linked to quantitative morphometric analysis. In addition, GT1-7 cells were transfected with mRFP-GFP-LC3 (Yoshii and Mizushima, 2017) and subsequently treated with 1 & 10.0 $\mu$M of Spd and Ril in the presence and absence of BafA1 and analysed with FM.

## 3.2 Effect of spermidine and rilmenidine on cell viability

In order to choose a suitable, yet non-toxic concentration of autophagy modulators, WST-1 was performed. For the concentrations assessed, no decrease in viability was observed. In fact, a minor metabolic response associated with enhanced reductive capacity was observed. Reductive capacity was significantly increased using 1 $\mu$M Spd $104.70\% \pm 1.64\%, p < 0.05$ and 10 10 $\mu$M Spd ($109.20 \pm 1.38\%, p < 0.05$) compared to the control ($100.00\% \pm 1.50\%$), with no significant differences observed using 0.1 $\mu$M Spd ($104.20\% \pm 1.46\%$). More importantly, a significant increase in reductive capacity was observed with 10 $\mu$M Spd ($p < 0.01$) compared to 0.1 $\mu$M Spd and 1 $\mu$M Spd.

Following rilmenidine treatment, a significant increase in reductive capacity was observed in the 10 $\mu$M Ril treated group ($105.70 \pm 2.26\%, p < 0.05$) compared to the control ($100.40 \pm 1.16\%$), with no significant differences observed in the 0.1 $\mu$M Ril ($98.76\% \pm 1.49\%$) and 1 $\mu$M Ril ($100.50 \pm 2.12\%$). Importantly, a significant increase in cellular viability was observed in the 10 $\mu$M Ril group compared to 0.1 $\mu$M and 1 $\mu$M Ril. This data suggests no toxicity and allowed to choose a low and high concentration of spermidine and rilmenidine for subsequent experiments.

## 3.3 Effect of spermidine and rilmenidine on autophagic flux using western blotting

To further identify the concentration that would have maximal autophagy inducing effects, protein extraction was performed following 8 hr treatment with a range of Spd and Ril

concentrations. Western blot analysis was performed to assess the protein expression of LC3-II and p62. LC3 II is formed through conjugation of LC3-I to PE and is localized on the inner and outer surface of the autophagosome membrane, where it is either degraded upon fusion of autophagosomes with lysosomes or removed through deconjugation and recycled (Kabeya *et al.*, 2000). Importantly, the abundance of LC3 II correlates with the number of autophagosomes and hence is used as a key marker to indicate autophagy or the size of the autophagosome pool (Loos *et al.*, 2014) p62 serves as a scaffolding for ubiquitinated proteins (Sahani *et al.*, 2014). It binds directly to LC3 and is in turn selectively degraded through autophagy; thus it is widely used as an additional indicator for autophagy activity (Pankiv *et al.*, 2007).

## Abundance of LC3 II and p62

A significant increase in the abundance of LC3 II was observed in the 10 $\mu$M Spd + BafA1 ($1.67 \pm 0.28$) compared to the control group ($1.00 \pm 0.00$). Moreover, a significant increase in the abundance of LC3 II was observed in the 10 $\mu$M Spd + BafA1 ($p < 0.05$) compared to0.1 $\mu$M Spd + BafA1. No significant differences were observed between 0.1 $\mu$M Spd, 1 $\mu$M Spd and 10 $\mu$M Spd. A significant increase was observed in the 10 $\mu$M Spd + BafA1 compared to 10 $\mu$M Spd, with no significant differences seen between 0.1 $\mu$M Spd and 0.1 $\mu$M Spd + BafA1 and between 1 $\mu$M Spd versus 1 $\mu$M Spd + BafA1, however it is important to mention that in both the 0.1 $\mu$M Spd and 1 $\mu$M Spd, inhibition of degradation with BafA1 resulted in an increased LC3 II accumulation compared to the Baf untreated groups, suggesting the presence of a basal as well as increased autophagic activity.

The abundance of p62 was significantly increased in the BafA1 treated group ($1.72 \pm 0.26, p < 0.05$) and 10 $\mu$M Spd + BafA1 ($1.72 \pm 0.17, p < 0.05$) compared to Con ($1.00 \pm 0.00$). Moreover, in comparison to the Bafa1 treated group, a significant decrease in p62 abundance was observed in the 1 $\mu$M Spd, with no significant differences seen in all other groups. A higher abundance was indicated in the presence of BafA1 in all treatment groups compared to the respective BafA1 untreated groups.

With regards to rilmenidine treatment, no significant differences in the abundance of LC3 II were observed, however an increasing trend in the amount of LC3 II in the BafA1 treated groups compared to the BafA1 untreated groups was noted [Con ($1.00 \pm 0.00$), BafA1 ($1.45 \pm 0.49$), 0.1 $\mu$M ($1.16 \pm 0.09$), 0.1 $\mu$M Ril + BafA1 ($1.46 \pm 0.36$), 1 $\mu$M Ril ($0.94 \pm 0.14$), 1 $\mu$M Ril + BafA1 ($1.22 \pm 0.42$), 10 $\mu$M Ril ($1.02 \pm 0.27$) and 10 $\mu$M Ril + BafA1 ($1.17 \pm 0.63$)].

A significant increase in p62 protein expression was observed in the 1 $\mu$M Ril ($1.68 \pm 0.19, p < 0.05$), 1 $\mu$M Ril + BafA1 ($2.21 \pm 0.31, p < 0.05$) and 10 $\mu$M Ril + BafA1 ($1.81 \pm 0.08, p < 0.05$) compared to the Con group ($1.00 \pm 0.00$). Moreover, a significant increase in p62 protein levels was revealed in the 1 $\mu$M Ril + BafA1 compared to BafA1 treated group. Furthermore, a significant increase in p62 abundance was observed

in the 1 $\mu$M Ril + BafA1 ($p < 0.05$) compared to 0.1 $\mu$M Ril + BafA1, with no significant differences observed when compared to 10 $\mu$M Ril + BafA1. Finally, overall p62 protein abundance accumulated in the BafA1 treated groups compared to the respective BafA1 untreated groups, however not reaching significance. No response was observed in 0.1 $\mu$M Ril + BafA1 compared to its BafA1 untreated group. This data suggests an autophagy enhancing effect, which is best revealed through the impacted p62 clearance.

## LC3 II and p62 protein turnover

# Chapter 4

# Role of spermidine and rilmenidine in a paraquat-induced neuronal toxicity model

## 4.1 Introduction

# Chapter 5

# Method development for Correlative Light and Electron Microscopy (CLEM)

## 5.1 Introduction

# Chapter 6

# Effect of spermidine on PQ induced brain injury model of Alzheimer's disease

## 6.1 Introduction

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